

# **EXHIBIT C**

## EXPERIMENTAL STUDY

# The Antioxidant N-2-Mercaptopropionyl Glycine Attenuates Left Ventricular Hypertrophy in In Vivo Murine Pressure-Overload Model

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<b>OBJECTIVES</b>	In order to identify the role of reactive oxygen species (ROS) in cardiac hypertrophy, we examined the effect of N-2-mercaptpropionyl glycine (MPG) on cardiac hypertrophy.
<b>BACKGROUND</b>	Recent in vitro studies have suggested that ROS play an important role as a second messenger in cardiac hypertrophy. It was therefore thought to be of particular value to examine the relevance of studies using in vitro models for cardiac hypertrophy in an in vivo setting.
<b>METHODS</b>	The transverse thoracic aorta in mice was constricted, and MPG (100 mg/kg) was infused intraperitoneally twice daily. The animals were assessed seven days after the operation for hemodynamic functions, oxidative stress and antioxidative enzyme activities.
<b>RESULTS</b>	Banding of the transverse aorta in mice resulted in an increase in the ratio of heart weight to tibia length and the appearance of an endogenous atrial natriuretic factor messenger ribonucleic acid (mRNA) seven days postoperatively. Administration of MPG significantly attenuated the hypertrophic responses induced by pressure overload. Cardiac hypertrophy was accompanied by increases in heme oxygenase-1 mRNA expression and lipid peroxidation, which was eliminated by the treatment with MPG. Pressure overload led to increases in antioxidant enzyme activities, such as superoxide dismutase and glutathione peroxidase, but not catalase, activity.
<b>CONCLUSIONS</b>	Our results indicated that oxidative stress was increased in our model and that it plays an important role in the development of cardiac hypertrophy. (J Am Coll Cardiol 2002;39: 907-12) © 2002 by the American College of Cardiology Foundation

Cardiac hypertrophy is an adaptive physiological process in response to various extracellular stimuli, such as mechanical stress, cytokines and growth factors. During the hypertrophic response, these stimuli activate intracellular signaling cascades, resulting in qualitative and quantitative changes in the contractile protein content and induction of an embryonic gene program (1). The hypertrophic response is compensatory in nature, but sustained excessive workloads may lead to heart failure. Epidemiological studies suggest that cardiac hypertrophy is an independent risk factor for cardiac morbidity and results in a significant increase in the risk of mortality from cardiovascular diseases (2,3). Fundamental advances in our understanding of the molecular basis of cardiac hypertrophy are expected to form the foundation for novel and more effective therapeutic and preventive approaches compared with those previously employed for patients with cardiac hypertrophy and heart failure.

It has been reported that reactive oxygen species (ROS)

contribute to myocardial cell damage and cardiac dysfunction (4-6). A growing body of evidence has suggested that ROS act as intracellular signaling molecules in stress response in a variety of cell types (7). Recently, ROS have been found to be involved in cardiac hypertrophy in neonatal or adult cardiomyocytes (8-10). Because these studies utilized cardiomyocyte models of hypertrophy, it would be of particular value to examine the relevance of studies using in vitro models to pressure overload hypertrophy in an in vivo setting.

In the study presented here, we examined the effects of an antioxidant on cardiac hypertrophy in an in vivo model (11) using the in vivo murine model of pressure overload hypertrophy. Pressure overload hypertrophy was introduced with the transverse thoracic aorta constriction (TAC) technique. This model is widely used for the study of transgenic and knockout mice in order to identify molecular mechanisms for cardiac hypertrophy. In this model, banding of the transverse aorta in mice leads to hyperfunctional hypertrophy after one week without showing any signs of heart failure (11). In this study, we showed that intraperitoneal injection of an antioxidant, N-2-mercaptpropionyl glycine (MPG), significantly attenuated cardiac hypertrophy induced by TAC in mice.

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#### Abbreviations and Acronyms

ANF	= atrial natriuretic factor
GSHPx	= glutathione peroxidase
HO-1	= heme oxygenase-1
MDA	= malonaldehyde
MPG	= N-2-mercaptopropionyl glycine
ROS	= reactive oxygen species
SOD	= superoxide dismutase
TAC	= transverse thoracic aorta constriction

## METHODS

This study was carried out under the supervision of the Animal Research Committee and in accordance with the Guidelines for Animal Experiments of Osaka University and the Japanese Animal Protection and Management Law (No. 25).

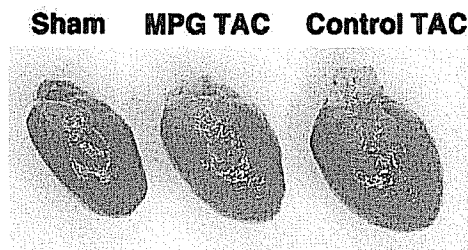
**Surgical procedures.** Ten-week-old male mice (C57/BL6) were anesthetized with a mixture of ketamine (100 mg/kg; intraperitoneal injection) and xylazine (5 mg/kg; intraperitoneal injection). TAC was performed as previously described (11). Briefly, the animals were intubated and ventilated under a dissecting microscope, with a small-animal respirator (model SN-480-7-10; Shinano Seisakusyo, Tokyo, Japan), at a rate of 110 cycles/min and a tidal volume of 1 ml/100 g body weight. Aortic constriction was performed by tying a 7-0 silk string ligature around a 26-gauge needle and then removing the needle. The chest was then closed and the mice were extubated and allowed to recover. Seven days after the aortic constriction, the mice were anesthetized, intubated and ventilated with the respirator as described above. The right and left carotid arteries were cannulated with heat-stretched PE 50 tubing combined with a pressure transducer (TP-300T; Nihon Kohden, Tokyo, Japan). The aortic pressure was digitized and processed with a computer system (model PE-1000; Nihon Kohden, Tokyo, Japan). After the pressure measurements, the heart was excised, weighed and frozen in liquid nitrogen.

**MPG treatment.** The mice were randomized to one week of treatment with either MPG (100 mg/kg, intraperitoneal injection) or an equal volume of its vehicle (phosphate buffered saline). The intraperitoneal injections of MPG were performed twice daily for seven days.

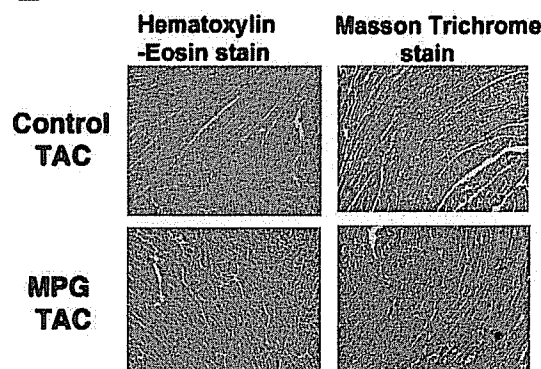
**Northern blot analysis.** Total ribonucleic acid (RNA) was extracted from the hearts with the aid of TRIZOL (Life Technologies, Inc., Rockville, Maryland), denatured in formaldehyde, fractionated on 1.0% agarose gel and transferred onto nitrocellulose membrane. The following complementary deoxyribonucleic acid (cDNA) probes were used: heme oxygenase-1 (HO-1); 640-bp ApaI and EcoRI fragment of the rat heme oxygenase, atrial natriuretic factor (ANF); 700-bp HindIII, BamHI fragment of the rat ANF.

**Lipid peroxidation.** The level of malonaldehyde (MDA) in left ventricles was measured as an index of lipid peroxidation as described previously (12) by using BIOXYTECH

**A**



**B**



**Figure 1.** Effect of N-2-mercaptopropionyl glycine (MPG) on cardiac hypertrophy induced by transverse aortic constriction (TAC). After the TAC procedure, mice received MPG twice daily intraperitoneally, were sacrificed seven days after TAC, and their hearts were removed and photographed (A). The hearts were histologically examined by hematoxylin-eosin and Masson trichrome stains (B). Control TAC = vehicle-treated TAC-operated mice; MPG TAC = MPG-treated TAC-operated mice; Sham = vehicle-treated sham-operated mice.

LPO-586 kit (Oxis International Inc., Portland, Oregon) according to the manufacturer's instructions.

**Antioxidant enzyme assay.** Superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) activity in the hearts was determined with the aid of BIOXYTECH SOD-225 and GPx-340 assay kit, respectively, according to the manufacturer's instructions (OXIS International, Portland, Oregon). Catalase activity was measured as described by Dhalla et al. (13).

**Statistical Methods.** Data are expressed as mean  $\pm$  SEM. Two-way analysis for variance (ANOVA) was used to test significance, with one factor being sham versus TAC operation and the other factor being vehicle versus MPG treatment. For variables with  $p < 0.05$  regarding the effect of interaction, we have analyzed the data among groups by one-way ANOVA with Tukey-Kramer's post hoc test for multiple comparisons. A  $p$  value  $< 0.05$  was accepted as statistically significant.

## RESULTS

**Physiologic and morphologic assessment of hypertrophy.** After seven days of chronic pressure overload, the mice showed a dramatic increase in heart size relative to sham-operated mice (Fig. 1). Treatment with MPG appeared to

**Table 1.** Effect of N-2-Mercaptopropionyl Glycine on Characteristics of Transverse Thoracic Aorta Constriction-Operated Mice

	Sham		TAC	
	Control (n = 7)	MPG (n = 7)	Control (n = 10)	MPG (n = 15)
HR (beats/min)	283 ± 20	276 ± 13	236 ± 9	280 ± 17
BW (g)	24.9 ± 0.46	24.8 ± 0.56	24.4 ± 0.36	24.5 ± 0.35
HW (mg)	126 ± 3.7	125 ± 2.9	145 ± 3.0*†	140 ± 3.2*†
LVW (mg)	84 ± 2.6	83 ± 2.4	105 ± 2.7*†	97 ± 2.1*†
TL (mm)	18 ± 0.07	18 ± 0.08	18 ± 0.05	18 ± 0.04
SBP (mm Hg)	89 ± 4.4	90 ± 3.7	120 ± 3.2§	123 ± 6.3§
PG (mm Hg)	1.2 ± 0.4	0.8 ± 0.3	30.1 ± 1.0§	31.1 ± 1.7§
LVW/BW (mg/g)	3.4 ± 0.10	3.4 ± 0.07	4.3 ± 0.10*†	3.9 ± 0.06*†‡
LVW/TL (mg/mm)	4.7 ± 0.15	4.6 ± 0.13	5.9 ± 0.16*†	5.4 ± 0.11*†‡

Data are expressed as mean value ± SEM. Two-way analysis for variance (ANOVA) was used to test significance, with one factor being sham versus TAC operation and the other factor being vehicle (control) versus MPG treatment. In the face of significant interactions between treatments and operations (HW, LVW, LVW/BW, and LVW/TL), one-way ANOVA was employed.

\*p < 0.05 versus control-vehicle group, †p < 0.05 versus sham-MPG group, ‡p < 0.05 versus TAC-control group, §p < 0.05 TAC versus sham-operated groups by two-way ANOVA.

BW = body weight; HR = heart rate; HW = heart weight; LVW = left ventricular weight; MPG = N-2-mercaptpropionyl glycine; PG = pressure gradient; calculated as the difference between right and left carotid artery systolic pressure; SBP = systolic blood pressure, proximal for TAC; TAC = transverse thoracic aortic constriction; TL = tibial length.

reduce the increase in heart size induced by pressure overload. As shown in Table 1, the average ratio of heart weight to tibia length, as well as that to body weight, was significantly higher for control TAC hearts than for control sham-operated hearts, but one week of treatment with MPG resulted in a significant attenuation of increases in those ratios by TAC. Heart rate, body weight and tibia length did not differ among groups. Although TAC procedure significantly increased systolic pressure and pressure gradients between the two carotid arteries, there were no significant differences in those between MPG- and vehicle-treated groups. In agreement with the previous report (11), the banded mice showed no signs of ischemic injury, necrosis or fibrosis in hearts (Fig. 1).

The reactivation of ANF gene expression in ventricular cells occurs in response to hypertrophic stimuli and is used as a marker of cardiac hypertrophy. Transverse thoracic aorta constriction led to a marked increase in ANF mRNA expression, as previously reported (Fig. 2), and treatment with MPG reduced this increase in ANF expression.

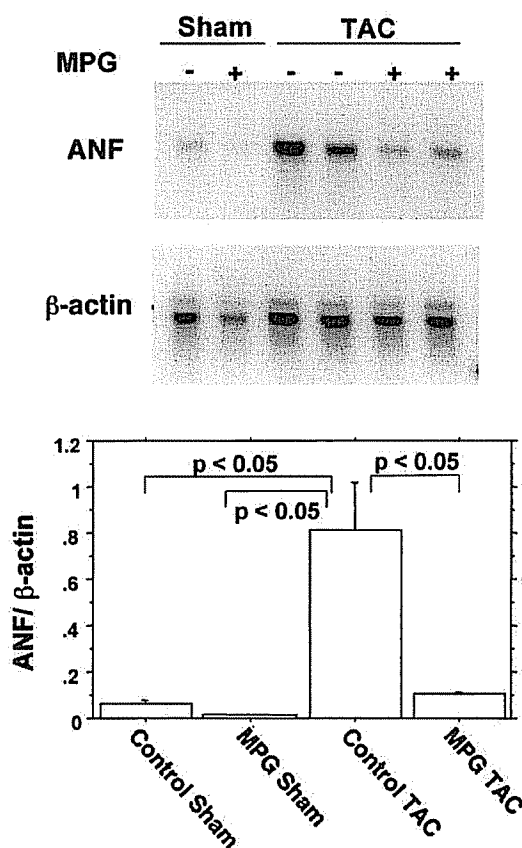
**Oxidative stress in hearts.** Heme oxygenase-1 is a stress response protein that is regulated by oxidative stress, and the mRNA expression of HO-1 has been used as a marker of the redox state (14). Northern blot analysis for HO-1 was performed to examine the effect of MPG on the redox state of the heart. As shown in Figure 3, thoracic aortic banding of the mouse aorta led to a marked increase in the expression of the HO-1 mRNA in the hypertrophied ventricle. This suggests that aortic banding induces ROS generation in hearts. N-2-mercaptpropionyl glycine significantly attenuated the increase in HO-1 expression, indicating MPG could scavenge the ROS generated by aortic banding. Western blot analysis indicated that the changes in HO-1 mRNA were paralleled by changes in protein expression (data not shown). To confirm this result, we examined the

effects of MPG on lipid peroxidation in hearts, which is used as an indicator of oxidative stress in cells and tissues. The concentration of lipid peroxide, estimated as MDA, increased in hypertrophied ventricle compared with that in the ventricle of the control sham-operated hearts (Fig. 4). Treatment with MPG inhibited the increase in MDA level induced by TAC. This suggested that chronic pressure overload caused oxidative stress in the heart and intraperitoneal injection of MPG reduced this oxidative stress.

**Changes in antioxidant enzymes.** The activities of antioxidant enzymes, including SOD, GSHPx and catalase, are known to change under various physiological and pathological conditions. Our measurement of the activities of superoxide dismutase, glutathione peroxidase and catalase showed that in the banded mice, myocardial SOD and GSHPx activities were higher than in the sham-operated mice (Fig. 5). N-2-mercaptpropionyl glycine treatment had no effect on myocardial SOD and GSHPx activities; catalase activity did not change significantly in any of the groups.

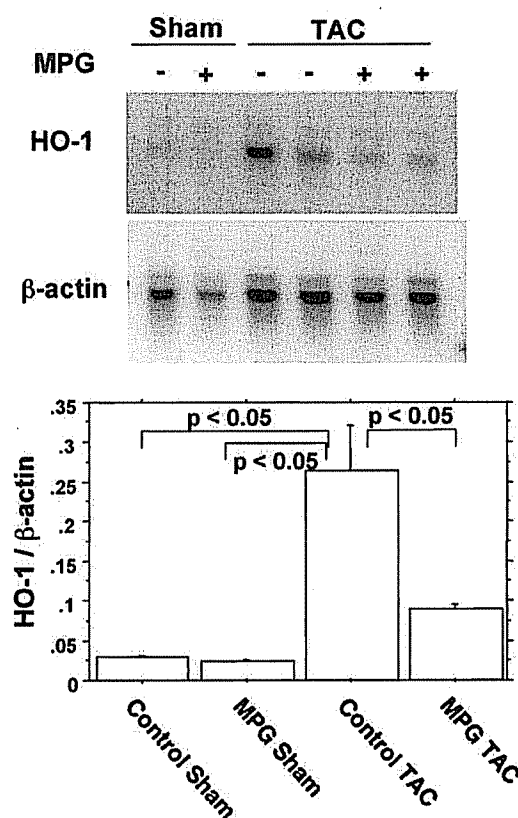
## DISCUSSION

**Level of oxidant stress in hypertrophic hearts.** Our study demonstrated that hypertrophied hearts show an increase in oxidative stress, as evidenced by higher lipid peroxidation and higher expression of HO-1 mRNA. The activities of antioxidant enzyme, including SOD, GSHPx and catalase, are known to change under various physiological and pathological conditions. Oxidative stress depends on the balance between endogenous antioxidant capacity and the amount of ROS. We were able to show that hypertrophy was associated with increases in SOD and glutathione peroxidase activities. The increase in the antioxidative enzyme activities in the hypertrophied hearts may be a



**Figure 2.** Effect of N-2-mercaptopropionyl glycine (MPG) on atrial natriuretic factor (ANF) mRNA expression. Total RNA was isolated from hearts treated with (+) or without (-) MPG and analyzed for expression of ANF mRNA by northern blot. A representative northern blot analysis result is shown in the upper panel. Sham = sham-operated mice; TAC = TAC-operated mice. Lower panel shows the quantitative analysis of ANF mRNA expression relative to  $\beta$ -actin mRNA based on densitometric measurements on northern blot. Data are expressed as mean  $\pm$  SEM (n = 3). Two-way analysis for variance (ANOVA) was used to test significance, with one factor being sham versus TAC operation and the other factor being vehicle versus MPG treatment. As we faced a significant interaction, we have analyzed the data among groups by one-way ANOVA and Tukey-Kramer's post hoc test.

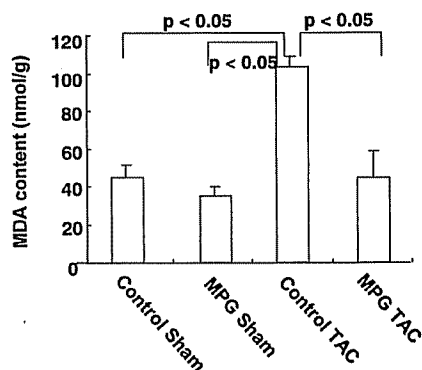
compensatory response to oxidative stress during cardiac hypertrophy. Our results made it clear that aortic banding increased the production of ROS and antioxidant reserves during cardiac hypertrophy. In our model, there might be a relative deficit in the antioxidant capacity of the myocardium to compensate for an increase in oxidative stress. Our findings agree with those reported by Duarte et al. (15) of an increase in plasma MDA level in spontaneously hypertensive rats. However, the hypertrophy of rat hearts induced by narrowing of the abdominal aorta for 4 to 48 weeks was reported to be accompanied by a decrease in the lipid peroxide content (16). The same authors reported that hypertrophied guinea pig hearts, induced by banding of the ascending aorta for 10 weeks, showed a decrease in lipid peroxidation as indicated by MDA content (17). In agreement with our results, there were significant increases in SOD and GSHPx activities at the hyperfunctional hyper-



**Figure 3.** Effect of N-2-mercaptopropionyl glycine (MPG) on heme oxygenase (HO)-1 mRNA expression. Total RNA was isolated from hearts treated with (+) or without (-) MPG and analyzed for expression of HO-1 by northern blot. A representative northern blot analysis result is shown. Sham = sham-operated mice; TAC = transverse aortic constriction (TAC)-operated mice. Lower panel shows the quantitative analysis of HO-1 mRNA expression relative to  $\beta$ -actin mRNA based on densitometric measurements on northern blot. Data are expressed as mean  $\pm$  SEM (n = 3). Control Sham = vehicle-treated sham-operated mice; control TAC = vehicle-treated TAC-operated mice; MPG sham = MPG-treated sham-operated mice; MPG TAC = MPG-treated TAC-operated mice. Two-way analysis for variance (ANOVA) was used to test significance, with one factor being sham versus TAC operation and the other factor being vehicle versus MPG treatment. As we faced a significant interaction, we have analyzed the data among groups by one-way ANOVA with Tukey-Kramer's post hoc test.

trophy stage in the banded animals (13,16,17). These studies also showed heart failure under chronic conditions was associated with reduced antioxidative capacity and increased oxidative stress. In our study, the banded mice showed no sign of heart failure estimated by echocardiography (data not shown). The antioxidant reserve may thus differ among experimental models.

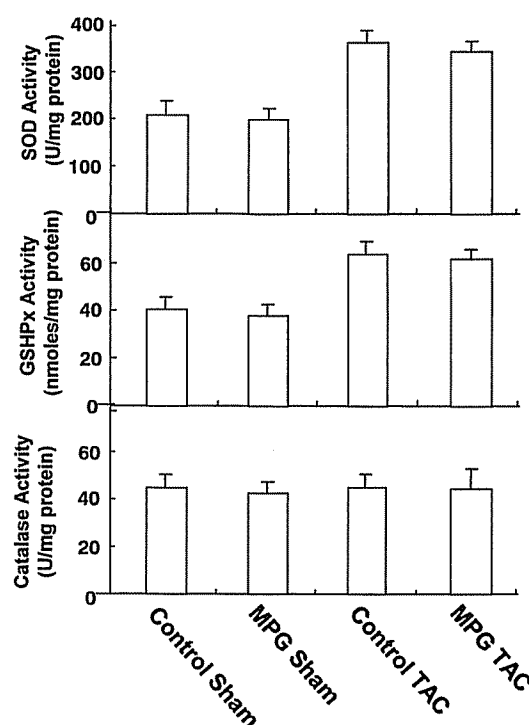
**The role of ROS in cardiac hypertrophy.** In this study, we showed that administration of MPG resulted in suppression of hypertrophy. This result agrees with in vitro data that antioxidants inhibited G-protein coupled receptor agonist-induced hypertrophy in rat neonatal cardiomyocytes (8,10). Although ROS play an important role in the pathogenesis of cardiac hypertrophy, some studies, as mentioned earlier, found that in hypertrophied hearts exhibiting



**Figure 4.** Effect of N-2-mercaptpropionyl glycine (MPG) on transverse aortic constriction (TAC)-induced changes in lipid peroxidation. One week after the operation, mouse hearts were homogenized and subjected to a lipid peroxidation assay ( $n = 4$ ). Control sham = vehicle-treated sham-operated mice; control TAC = vehicle-treated TAC-operated mice; MDA = malonaldehyde; MPG sham = MPG-treated sham-operated mice; MPG TAC = MPG-treated TAC-operated mice. Data are expressed as mean  $\pm$  SEM. Two-way analysis for variance (ANOVA) was used to test significance, with one factor being sham versus TAC operation and the other factor being vehicle versus MPG treatment. As we faced a significant interaction, we have analyzed the data among all combination of groups by one-way ANOVA with Tukey-Kramer's post hoc test.

a reduction in oxidative stress, vitamin E treatment did not have a significant effect on hypertrophy or on the content of thiobarbituric acid reactive substance (13). The differences in antioxidant reserve among experimental modes may explain the discrepancy regarding the effect of antioxidants on cardiac hypertrophy. In our study, MPG treatment resulted in almost complete elimination of increases in the MDA content and HO-1 expression, but only a partial inhibition of the increase in the LV/tibia ratio. These findings suggest that both ROS-dependent and -independent signaling pathways are present in the signal transduction system in cardiac hypertrophy.

The half-time of MPG is reported to be  $<7$  min (18), and we injected MPG twice daily. This suggested that MPG administration might result in a periodic reduction in ROS level. The molecular mechanism underlying decreases in MDA level and HO-1 mRNA expression, and inhibition of cardiac hypertrophy by the periodic reduction in ROS level remains to be elucidated. However, there might be a possible mechanism to account for these results. Cardiac hypertrophy and heart failure are frequently accompanied by elevated plasma levels of TNF- $\alpha$  (19,20). TNF- $\alpha$  is thought to contribute to cardiac hypertrophy via the generation of ROS (8), whereas ROS induce TNF- $\alpha$  in hearts (21). Taken together, these points suggest there may be a positive-feedback mechanism for ROS generation in cardiomyocytes. Augmented ROS generation in a positive-feedback manner might be necessary to induce HO-1 expression, MDA formation and cardiac hypertrophy. N-2-mercaptpropionyl glycine injection, which was performed twice daily, might be enough to inhibit the augmentation of ROS generation.



**Figure 5.** Effect of N-2-mercaptpropionyl glycine (MPG) on transverse aortic constriction (TAC)-induced changes in antioxidant enzyme activities. One week after the operation, mouse hearts were homogenized and subjected to superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase activity assays ( $n = 4$ ). Control sham = vehicle-treated sham-operated mice; control TAC = vehicle-treated TAC-operated mice; MPG sham = MPG-treated sham-operated mice; MPG TAC = MPG-treated TAC-operated mice. Data are expressed as mean  $\pm$  SEM. Two-way ANOVA indicated significant difference between TAC versus sham-operated mice, but not vehicle and MPG-treated mice in SOD and GSHPx activities without significant interaction. There were no significant differences in catalase activity between TAC and sham-operated groups and between vehicle and MPG-treated groups.

On the other hand, the periodic reduction in ROS level by MPG was not sufficient to inhibit the activations of SOD and GSHPx. Thus, the amount and time course of ROS generation appropriate for the signal transduction system might be different among cellular responses.

**Study limitations.** In this study, we employed an in vivo murine model of pressure overload hypertrophy introduced with TAC. An abrupt increase in pressure was loaded to hearts, and hypertrophy was completed in a week. Thus, the time course of hypertrophy was far different from that in clinically observed cardiac hypertrophy in humans, which means that our findings may not be generalized to human patients with cardiac hypertrophy. To assess the effectiveness of the antioxidant therapy to cardiac hypertrophy, long-term prognostic power must be demonstrated in human population.

**Conclusions.** In conclusion, we demonstrated that the level of oxidative stress increases in an in vivo murine model of pressure overload hypertrophy, and that antioxidant therapy inhibited cardiac hypertrophy.

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